

Universitat de Lleida

Document downloaded from:

<http://hdl.handle.net/10459.1/59387>

The final publication is available at:

<https://dx.doi.org/10.1016/j.scienta.2017.02.039>

Copyright

cc-by-nc-nd, (c) Elsevier, 2017



Està subjecte a una llicència de [Reconeixement-NoComercial-SenseObraDerivada 4.0 de Creative Commons](https://creativecommons.org/licenses/by-nc-nd/4.0/)

Manuscript Number: HORTI18059R1

Title: REFRIGERATED STORAGE AND CALCIUM DIPS IN RIPE 'CELESTE' SWEET CHERRY FRUIT: COMBINED EFFECTS ON CELL WALL METABOLISM.

Article Type: Research Paper

Section/Category: Tree crops, Fruit, Postharvest biology and storage studies, Tree and fruit physiology

Keywords: Calcium; cell wall; enzymes; firmness loss; gene expression; *Prunus avium* L.; sweet cherry

Corresponding Author: Dr. Isabel Lara Ayala, PhD

Corresponding Author's Institution: UdL

First Author: Burcu Belge

Order of Authors: Burcu Belge; Luis F Goulao; Eva Comabella; Jordi Graell; Isabel Lara Ayala, PhD

Abstract: Ripening-related firmness loss shortens considerably the storage potential of sweet cherry (*Prunus avium* L.), thus limiting postharvest handling, transportation, and commercialisation. The biochemical mechanisms underlying this process in cherries are not fully understood, and the mechanisms operating in a given fruit may be not extrapolated to a different species. Cell wall materials obtained from untreated and calcium-treated 'Celeste' sweet cherries were fractionated and analysed after cold storage, and related enzyme activities and gene expression were assessed. Calcium-treated fruit were firmer, with lower weight loss and decay incidence than the controls. The accumulation of Pa β Gal and PaEXP1 transcripts was strongly inhibited in cold-stored fruit, although expression levels recovered largely after three days of shelf life. Data suggest that pectin methylesterase, β -galactosidase and expansin activities may control the access of additional proteins to their substrates. A possible role for the apoplastic redox status in the modulation of the process is also discussed.



Universitat de Lleida
Departament de Química

Av. Alcalde Rovira Roure, 191
E 25198 LLEIDA (Catalunya)
Tel. +34 973 70 26 66
Fax +34 973 23 82 64
secretaria@quimica.udl.cat
<http://www.etsea.udl.cat/dept/quimica>

Lleida, February 17th, 2017

John Patrick Bower, PhD

Editor

SCIENTIA HORTICULTURAE

Dear Dr. Bower,

Please find attached a revised version of our manuscript HORTI18059, entitled '*Refrigerated storage and calcium dips in ripe 'Celeste' sweet cherry fruit: Combined effects on cell wall metabolism*', by Belge et al. We have modified slightly the original manuscript in response to the remarks raised by the Reviewer, and we hope it is now suitable for publication in SCIENTIA HORTICULTURAE. An explanation of the changes undertaken is attached to this letter.

I acknowledge you for your attention, and look forward to your decision.

Sincerely yours,

Dr. Isabel Lara Ayala
Departament de Química
Universitat de Lleida

We are enormously grateful to the Reviewer and the Editor for consideration of our manuscript, and for the kind, positive comments on our work. Responses to the Reviewer's suggestions are given below.

Reviewer # 1

1. Differences in SSC, TA and SSC/TA ratios are now described in lines 221-223. Decay data are mentioned in lines 235-237.
 2. The Reviewer suggests considering if the Results and Discussion section could be shortened. We have indeed gone through the text, and removed a couple of lines here and there. However, we find it very difficult to shorten this section appreciably without loss of significance. The reasons are the complexity of the process of cell wall disassembly, the many variables assessed, and the difficulty to address all results comprehensively.
 3. The Reviewer also suggests rotating Tables 2 to 5 90° clockwise, because they might be too big and hard to edit as they stand presently. We have no objections at all in doing so, and it would be easy indeed. Nevertheless, after considering the Tables, we have noticed that Table 2 would end up having 11 columns, which is wider than at present. The rest of Tables would have 8 or 9 columns, which does not differ significantly in relation to the current format. For Table 3 uniquely would this change result in a narrower design, but we would prefer that all Tables share the same format. We are hence undecided as to this point, and will submit to the Editor's opinion.
-

1 **Refrigerated storage and calcium dips in ripe ‘Celeste’ sweet cherry**
2 **fruit: Combined effects on cell wall metabolism**

3

4 Burcu Belge, Luis F. Goulao, Eva Comabella, Jordi Graell, Isabel Lara*

5

6 **Highlights**

7

- 8 • Cell wall metabolism was assessed in calcium-dipped cherries after cold storage
- 9 • Treated fruit were firmer, and had lower weight loss and decay incidence
- 10 • Both procedures affected gene expression and activity levels of some proteins
- 11 • Pectin methylesterase and β -galactosidase activity may be key for cherry
- 12 softening
- 13 • Expansin action and apoplastic redox status may also be pivotal for the process

**REFRIGERATED STORAGE AND CALCIUM DIPS IN RIPE
‘CELESTE’ SWEET CHERRY FRUIT: COMBINED EFFECTS ON
CELL WALL METABOLISM**

Burcu Belge¹, Luis F. Goulao^{2,†}, Eva Comabella¹, Jordi Graell¹, Isabel Lara^{1,*}

¹ Universitat de Lleida (UdL), Unitat de Postcollita-XaRTA, AGROTÈCNIO, Alcalde Rovira
Roure 191, 25198 Lleida, Spain.

² Instituto de Investigação Científica Tropical (IICT), Agri4Safe/BioTrop, Polo Mendes
Ferrão, Edifício Ferreira Lapa, Tapada da Ajuda, 1349-017 Lisboa, Portugal.

† Present address: Universidade de Lisboa, Instituto Superior de Agronomia, LEAF, Tapada
da Ajuda, 1349-017 Lisboa, Portugal.

* Corresponding author: **Isabel Lara**

e-mail: lara@quimica.udl.cat; Tel: +34 973 702526

Refrigerated storage and calcium dips in ripe ‘Celeste’ sweet cherry fruit: Combined effects on cell wall metabolism

Burcu Belge, Luis F. Goulao, Eva Comabella, Jordi Graell, Isabel Lara*

Abstract

Ripening-related firmness loss shortens considerably the storage potential of sweet cherry (*Prunus avium* L.), thus limiting postharvest handling, transportation, and commercialisation. The biochemical mechanisms underlying this process in cherries are not fully understood, and the mechanisms operating in a given fruit may be not extrapolated to a different species. Cell wall materials obtained from untreated and calcium-treated ‘Celeste’ sweet cherries were fractionated and analysed after cold storage, and related enzyme activities and gene expression were assessed. Calcium-treated fruit were firmer, with lower weight loss and decay incidence than the controls. The accumulation of *PaβGal* and *PaEXPI* transcripts was strongly inhibited in cold-stored fruit, although expression levels recovered largely after three days of shelf life. Data suggest that pectin methylesterase, β -galactosidase and expansin activities may control the access of additional proteins to their substrates. A possible role for the apoplastic redox status in the modulation of the process is also discussed.

Keywords: Calcium; cell wall; enzymes; firmness loss; gene expression; *Prunus avium* L.; sweet cherry

1. Introduction

Sweet cherry (*Prunus avium* L.) is a high-value stone fruit species, but displays limited storage and shelf life potential. Post-harvest life of these fruit is restricted by rapid firmness and weight loss, associated to high susceptibility to rots and mechanical damage. Modifications in the polymers and their cross-links on primary cell walls and middle lamella polysaccharides are generally regarded as a major driver of ripening-related fruit softening. These alterations eventually lead to decreased cell-to-cell adhesion and mechanical resistance of the pericarp tissue of fruit. These processes are facilitated by biochemical changes in the apoplast which alter the mobility and the activity of a complex set of cell wall-modifying proteins (Goulao and Oliveira 2008).

Pectin solubilisation is an early event in ripening-related fruit softening, followed in some species by pectin depolymerisation. Pectin solubilisation is reflected by an increase in the amounts of pectins loosely bound to the cell walls, recovered mainly in the water- and chelator-soluble fractions of the cell walls, often accompanied by decreased yields of covalently-bound materials. These modifications result largely from the coordinated action of a wide range of related proteins, both pectolytic and non-pectolytic. Previous studies suggest that depolymerisation of the pectin backbone in sweet cherry is limited (Batisse et al. 1994), texture differences in these fruit being related rather to the degree of loss of sugar residues from pectin side-chains (Batisse et al. 1996; Salato et al. 2013), which may be related to solubilisation. Earlier work has also shown that firmness of some, albeit not of all, cultivars of sweet cherry fruit may be also related partially to the amount and structure of cell wall matrix glycans, particularly of xyloglucans (Choi et al. 2002), the main hemicellulose component of cell walls. Indeed, xyloglucan endotransglycosylase/hydrolase (XTH) has been demonstrated to play a key role in ripening-related depolymerisation of hemicelluloses in fruit species such

as tomato (*Solanum lycopersicon* L.) and apple (*Malus × domestica* Borkh.) (Muñoz-Bertomeu et al. 2013).

In addition to pectolytic and non-pectolytic enzyme activities, ripening-related expansins have been identified and shown to be involved in the softening process of fruit species such as strawberry (*Fragaria × ananassa* Duch.) (Civello et al. 1999; Harrison et al. 2001), tomato (Brummell et al. 2002) or peach (*Prunus persica* L.) (Hayama et al. 2003). Expansins are responsible for loosening non-covalent linkages between cell wall polymers, and are thus considered an important contributor to firmness loss. For instance, a close relationship between Exp1 action and hemicellulose depolymerisation was found during tomato fruit softening (Brummell et al. 1999). However, expansin gene expression was not correlated to firmness loss in ‘Yumyeong’ peaches (Hayama et al. 2000), and therefore species- and cultivar-related variability cannot be ruled out.

Calcium is required for several key physiological processes connected to ripening-related phenomena, including those associated to the structure and functionality of cell wall and membrane, or to the activity of particular enzymes. Firmness retention is frequently cited as a major general effect of calcium applications in fruit, arising mostly from decreased pectin solubilisation and matrix glycan breakdown (reviewed in Lara 2013). Since calcium favours the establishment of electrostatic cross-links between polyuronides, calcium treatments (applied mostly as the chloride salt) help preventing the dissolution of the middle lamella, and accordingly often result in increased yields of the chelator-soluble fraction of pectins and better retention of total uronic acids. In the case of sweet cherry, a few studies have reported the effects of pre- and post-harvest calcium treatments in cultivars such as ‘0900 Ziraat’, ‘Lambert’, ‘Van’, ‘Merton Glory’, ‘Vega’, ‘Sue’, ‘Sweetheart’ or ‘Lapins’ (Demirsoy and Bilgener 1998; Vangdal et al. 2008; Wang et al. 2014). Higher fruit firmness, reduced cracking incidence and improved shelf life potential have been generally observed in treated

fruit. However, no significant treatment effects on firmness were found for the yellow-fleshed cultivars ‘Merton Glory’, ‘Vega’ and ‘Sue’ (Vangdal et al. 2008), showing cultivar-to-cultivar variation. In no case, though, were cell wall analyses undertaken in treated fruit, and thus the effects of calcium application on firmness-related factors were not examined at the biochemical level. Therefore, in this work we investigated the effects of post-harvest calcium dips on cell wall metabolism both at harvest and after refrigerated storage for two weeks in ‘Celeste’ cherries, a red-fleshed cultivar.

2. Materials and methods

2.1. Plant material, postharvest treatments, and analysis of standard quality

Uniform, defect-free ‘Celeste’ cherry (*Prunus avium* L.) fruit were hand-collected in 2012 (May 31st) from a family-led orchard located in Corbins (Lleida, NE Spain), at commercial maturity on the basis of size and surface colour, according to the usual standards in the producing area. Samples were transported immediately to the ETSEA-UdL campus, where they either remained untreated, or were dipped in 1.5 or 3% (w/v) CaCl₂ (2 min, room temperature) in the presence of 2% (w/v) NaClO as a disinfectant, and thereafter allowed to dry in air. Treated and untreated fruit were then stored at 0 °C and 92% relative humidity under regular air for two weeks. Fruit were analysed immediately after harvest and upon removal from storage, with three additional days at 20 °C to simulate commercial shelf life. Tissue samples were taken in triplicate from fruit pericarp (10 fruit per replicate) at each analysis date, frozen in liquid nitrogen, freeze-dried, powdered, and kept at -80 °C for subsequent biochemical analyses.

The experimental procedures for the analysis of standard quality attributes were undertaken as described in Belge et al. (2015). Fruit firmness and incidence of fungal decay were assessed on 30 fruit per treatment. A Durofel DFT 100 digital durometer (Agrosta Sarl,

Serqueux, France) was used for firmness measurements on two opposite sides of each individual fruit, and results given as Durofel units (1, no resistance - 100, maximum resistance). Stored fruit were allowed to temperate at room temperature for 3 hours prior to firmness measurements. The incidence of fungal decay was expressed as percentage of fruit affected. Respiration rates were measured as CO₂ produced ($\mu\text{g kg}^{-1} \text{ s}^{-1}$) by keeping fruit (3 replicates \times 10 fruit/replicate) into 3-L flasks aerated continuously with humidified air (25 mL s^{-1}), and then connecting the effluent air to an infrared gas analyser (Sensotran IR, El Prat de Llobregat, Spain). Three replicate samples (10 fruit each) per treatment were stoned and squeezed until no more juice was released. After filtration, the volume of juice recovered was considered as fruit juiciness (mL kg^{-1}). This juice was used to determine soluble solids content (SSC) and titratable acidity (TA), and data were expressed as % (w/w).

2.2. *Extraction, fractionation and analysis of cell wall materials*

Lyophilised pericarp tissue (3 g) was extracted in phenol:acetic acid:water (2:1:1, w/v/v) (PAW) in order to isolate the cell wall materials (CWM) as described elsewhere (Redgwell et al. 1992). The PAW-insoluble pellet was washed once in water and twice in acetone, and then lyophilised and weighed to determine CWM yield (g kg^{-1}) on a dry weight basis. For further fractionation, CWM samples (100 mg) were extracted sequentially in water, 0.05 mol L⁻¹ cyclohexane-trans-1,2-diamine tetra-acetate (CDTA), 0.05 mol L⁻¹ Na₂CO₃ and 4 mol L⁻¹ KOH (Selvendran and O'Neill 1987). All four CWM fractions obtained were filtered through Miracloth, dialysed (mol. wt. cut-off 7000), lyophilised and weighed, and yields expressed over total isolated CWM as g kg^{-1} .

Samples (30-35 mg) of total CWM, CDTA- and Na₂CO₃-soluble fractions were analysed for the content of uronic acids and total neutral sugars by the *m*-hydroxydiphenyl method and the phenol-sulphuric acid assay, respectively, after hydrolysis with 12 mol L⁻¹ sulphuric acid

as described elsewhere (Ortiz et al. 2011a). Neutral sugar contents in the samples were estimated by subtracting the content of uronic acids from that of total sugars. Data are given as g kg^{-1} on a dry weight basis.

The contents of reduced (AA) and oxidised (DHA) ascorbic acid were measured in lyophilised pericarp tissue (40 mg) as well as in total CWM (25 mg), using the colorimetric ascorbate assay (Gillespie and Ainsworth 2007), and data were given as mmol kg^{-1} . Calcium content in lyophilised pericarp (1g) and in samples (30-40 mg) of CWM was also analysed by inductively coupled plasma emission spectroscopy (ICP-OES) as in previous studies (Ortiz et al. 2011a), and results expressed as g kg^{-1} on a dry weight basis (**Fig. 1**) and over total isolated CWM (**Supplementary Fig. S1**).

2.3. Assay of cell wall-modifying enzyme activities

Enzyme assays included the pectolytic activities of poligalacturonase (exo-PG; EC 3.2.1.67 and endo-PG; EC 3.2.1.15), pectin methylesterase (PME; EC 3.1.1.11), pectate lyase (PL; EC 4.2.2.2), β -galactosidase (β -Gal; EC 3.2.1.23) and α -L-arabinofuranosidase (AFase; EC 3.2.1.55), as well as the non-pectolytic endo-1,4- β -D-glucanase (EGase; EC 3.2.1.4) and β -xylosidase (β -Xyl; EC 3.2.1.37) activities. For PG, PME, PL and EGase activities, extraction buffer contained 20 mmol L^{-1} Cys-HCl, 20 mmol L^{-1} EDTA and 0.05% (v/v) Triton X-100 in 20 mmol L^{-1} Tris-HCl at pH 7.0. For the extraction of AFase activity, buffer was composed of 100 mmol L^{-1} NaCl, 2% (v/v) β -mercaptoethanol and 1% (w/v) PVPP in 100 mmol L^{-1} acetate buffer at pH 5.2. β -Gal and β -Xyl activities were extracted in buffer containing 1 mol L^{-1} NaCl and 1% (w/v) PVPP in 50 mmol L^{-1} acetate at pH 6.0. A 10% (w/v) homogenate was obtained in each case by homogenising freeze-dried pericarp tissue (100 mg) in the corresponding extraction buffer. After centrifugation, the crude extract was set on ice, and the activity assays were undertaken as referenced in Ortiz et al. (2011a). One

unit (U) of PG activity was defined as the liberation of 1 μmol of GalUA min^{-1} from apple pectin. One unit (U) of PME or PL activity were defined as the decrease of one unit of $A_{620} \text{ min}^{-1}$ or the increase of one unit of $A_{235} \text{ min}^{-1}$, respectively, with apple pectin as the substrate. One unit (U) of EGase activity was defined as the release of 1 μmol of glucose min^{-1} from carboxymethylcellulose. One unit (U) of β -Gal or β -Xyl activity was defined as the liberation of 1 μmol of *p*-nitrophenol min^{-1} from *p*-nitrophenyl- β -D-galactopyranoside or 1 nmol of *p*-nitrophenyl- β -D-xylopyranoside, correspondingly. One unit (U) of AFase was defined as the liberation of 1 nmol of *p*-nitrophenol min^{-1} from *p*-nitrophenyl- α -L-arabinofuranoside. Total protein content was determined with the Bradford (1976) method, with BSA as a standard. Results were given as specific activity over total protein (U kg^{-1}).

2.4. RT-qPCR analysis of cell wall-related gene expression

Lyophilised pericarp tissue (250 mg) was used for the isolation of total RNA according to Chang et al. (1993). RNA concentration and purity were verified in a Synergy microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) equipped with the Gen5™ Data Analysis software. Samples of total RNA thus recovered were digested with RNase-free DNase, and cDNA was synthesised from DNA-free RNA (220 ng) by retrotranscription with oligo-(dT)₁₈ (Invitrogen, Waltham, MA, USA). A control standard RT-PCR was performed to verify each reaction using primers for the targeted genes, followed by the observation of a single band on 1.2% (w/v) agarose gels after electrophoresis of the products.

Primers for quantitative real time-PCR (RT-qPCR) amplification of the target genes (**Table 1**) were designed after search in NCBI databases using the Primer3 online software. Primer length was set to be 20 to 23 bp, GC content 45 to 65%, and melting temperature (T_m) 60 to 64 °C. Amplicon length was fixed to range between 80 and 120 bp. The OligoEvaluator™ (Sigma-Aldrich Co. LLC, St. Louis, MO, USA) online oligonucleotide

sequence calculator was then used to check the probability of formation of hairpin structures and of primer dimerization.

RT-qPCR reactions were prepared in 96-well plates, and run in a qTOWER 2.2 quantitative thermal cycler (Analytikjena, Germany). Total reaction volume was 20 μ L, containing 150 ng cDNA and 3 μ M each primer in 1 \times SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). Reaction running conditions were as follows: 95 °C during 30 s; then 40 cycles at 95 °C for 5 s, and 60 °C for 30 s. Dissociation curves were generated for each amplicon at the end of the PCR run by continuous fluorescence measurement (55 °C to 95 °C, with sequential steps of 0.5 °C for 15 s), in order to verify amplification specificity and the absence of primer dimers. Each reaction was run at least in triplicate, and the mean threshold cycle (Ct) was used for data analyses.

PCR amplification efficiencies were calculated for each primer pair. Five-fold serial dilutions spanning five orders of magnitude (1:1-1:625) of cDNAs pooled from all technical replications of each reaction were quantified to generate standard curves. PCR efficiencies were determined for each gene from the logarithm of the cDNA dilution plotted against the mean Ct values, using the equation: $E(\%) = (10^{-(1/\text{slope})} - 1) \times 100$, where E is the efficiency, in percentage, and slope is the gradient of the best-fit line in the linear regression.

Expression data were normalised by the geometric mean of the expression levels of three reference genes (*PaSRP19*, *PaPP2A-2* and *PaCAC*) selected on the basis of a published report (Alkio et al. 2012). All procedures for the calculation of amplification efficiencies and for the normalisation of relative expression values were as in earlier work (Vandesompele et al. 2002; Goulao et al. 2012).

2.5. Statistical analysis

All data were tested by analysis of variance (ANOVA), using a multi-factorial design with treatment, cold storage and shelf life period as the factors. These analyses were carried out with the Minitab 16 statistical software (Minitab Inc., State College, PA, USA). Means were separated by the Fisher's LSD test at $P \leq 0.05$.

3. Results and Discussion

The usual standard indicators of maturity and commercial quality were assessed in fruit at harvest and after storage (**Table 2**). Calcium dips were successful in increasing total calcium content in fruit pericarp, the amounts in treated samples being roughly two-fold those in the controls (**Table 3**). Treated fruit displayed higher respiration rates upon removal from storage in comparison with untreated controls, but rates levelled down after three days at 20 °C. Weight loss was substantial, with some associated decrease in fruit size, but significantly lower fruit dehydration was observed for samples treated with the highest (3%, w/v) calcium concentration, both 0 and 3 d after transfer to 20 °C (**Table 2**), which accordingly retained higher juiciness values. These fruit also displayed higher SSC/TA ratios, particularly upon removal from cold storage, owing to higher SSC values than those in samples either untreated or treated with a lower CaCl_2 dose, which suggests better retention of eating quality.

Fruit firmness increased significantly 3 d after harvest, which may relate to high fruit dehydration (roughly 12%) (**Table 2**). However, fruit firmness also increased after remaining two weeks at 0 °C in comparison with values at harvest. Whereas this observation may also be related to weight loss (around 7% upon removal from cold storage), it could also have arisen from a tightening effect of low temperature on fruit tissues, as found likewise for strawberries (Lara et al. 2004). Cherries treated with 3% CaCl_2 showed the highest firmness levels, together with the lowest weight loss, whereas no significant differences with the controls were observed for fruit treated with 1.5% CaCl_2 (**Table 2**). When samples were placed at 20 °C for

three days following cold storage, they softened appreciably, but calcium-treated cherries remained firmer. Although the highest firmness levels were found for fruit treated with 1.5% CaCl_2 , no differences with the controls were found regarding weight loss, which however was lower in fruit dipped in 3% CaCl_2 . Calcium-treated fruit also displayed significantly lower spoilage rates, both 0 and 3 d after being transferred to 20 °C, and therefore these treatments apparently exerted a protective effect against fungal decay. Cell wall materials were isolated and fractionated in an attempt to gain insight into the modifications induced in response to the factors considered herein.

3.1. Cell wall modifications after cold storage

The yield of insoluble cell wall materials (CWM) obtained in each case was expressed both on a dry weight basis (**Table 4A**) and as the relative amount over total CMW recovered from fruit pericarp (**Table 4B**). No consistent relationship was observed between total CWM yields and fruit firmness or decay incidence, thus indicating that this parameter is not a reliable predictor for these quality attributes. Yet when insoluble materials were fractionated, some significant differences were apparent in fraction yields in response to the factors considered.

Significantly increased yields of all four CWM fractions isolated were observed when unrefrigerated fruit were kept at 20 °C for three days. Changes in the chelator-soluble fraction (CDTA_{sf}), enriched in pectins linked non-covalently to the cell wall, underlay this augment, since the contents of this fraction raised 1.7-fold in comparison with those at harvest, while no significant variation was found for the rest of the fractions obtained (**Table 4A**).

Once fruit were removed from cold storage, CDTA_{sf} contents also showed substantial modifications in response to the treatments applied, levels in treated samples being two-fold those in untreated controls, and leading to higher values for the total amount of insoluble

materials recovered, both in absolute (**Table 4A**) and relative (**Table 4B**) terms. Solubilisation of cell wall materials was partially inhibited during exposure to cold temperature, as shown by significantly lower yields of water-soluble materials, as well as by higher contents of total insoluble polysaccharides in these samples as compared with unrefrigerated fruit. The contents of the sodium carbonate-soluble fraction ($\text{Na}_2\text{CO}_{3\text{sf}}$), corresponding mainly to covalently-bound pectins, showed no consistent relationship to fruit firmness, as fruit treated with 3% CaCl_2 yielded lower amounts of this fraction than untreated samples or than samples treated with 1.5% CaCl_2 , in spite of higher firmness levels, and lower weight loss and decay incidence in these fruit (**Table 2**).

After refrigerated fruit were left at 20 °C during three days, the amount of total insoluble materials recovered was even higher than at day 0, particularly for samples dipped in 3% CaCl_2 , in which CDTA_{sf} yields represented over 25% of total CWM (**Table 4B**). This may be indicating improved capacity for calcium-bridging between adjacent polymers as a consequence of higher availability of the cation in the cell walls (**Table 3**), which would reinforce and thus protect cell walls against extensive disassembly. Yet, again, no consistent relationship was found between CDTA_{sf} yields and fruit firmness (**Table 2**), which agrees with previous findings for strawberry (Lara et al. 2004) and peach (Ortiz et al. 2011b). The highest $\text{Na}_2\text{CO}_{3\text{sf}}$ yields, though, were found for samples treated with 1.5% CaCl_2 , together with the lowest amounts of solubilised materials (**Table 4**), and coincident with better retention of firmness in these fruit after remaining three days at 20 °C (**Table 2**).

When the chelator- and Na_2CO_3 -soluble fractions were analysed for the total content of neutral sugars and uronic acids, it was found that the changes in CDTA_{sf} yields (**Table 4A**) were in good accordance with those in the amounts of both types of sugar compounds in that fraction (**Fig. 1**). Indeed, the CDTA_{sf} isolated from calcium-dipped fruit contained significantly higher amounts in comparison with untreated samples. For instance, cherries

treated with 3% CaCl_2 contained more than double the amount of neutral sugars than the controls (2.91 vs. 1.22 g kg^{-1}) upon removal from cold storage, and similar differences between treated and untreated fruit were observed for uronic acids (2.25 vs. 0.97 g kg^{-1} , in the same order) (**Fig. 1A**). Samples treated with 1.5% CaCl_2 also showed significantly higher levels of both sugar types as compared with untreated fruit, although the differences were not so substantial. After three days at 20 °C, though, albeit cherries treated with 3% CaCl_2 still retained higher amounts of both neutral sugars and uronic acids, those dipped in 1.5% CaCl_2 displayed lower levels than those in the controls (**Fig. 1A**). Interestingly, these same samples also displayed the highest contents of uronic acids in the Na_2CO_3 -soluble fraction, both 0 and 3 d after removal from cold storage (**Fig. 1B**). Generally speaking, lower amounts of uronic acids in the carbonate-soluble fraction were accompanied by higher contents in the CDTA-soluble fraction, consistent with the idea that a part of the solubilised cell wall materials are reallocated transiently to the chelator-soluble fraction during the process of cell wall disassembly.

3.2. Cell wall-related enzyme activities after fruit storage

It is usually challenging to perceive clear trends and to draw conclusions when trying to relate ripening-related cell wall changes to the activity of cell wall-acting proteins, both enzymatic and non-enzymatic. These difficulties arise to a great extent from the close co-operation among the large number of involved proteins, as well as from the co-existence of multiple isoforms all of which contribute to the measured activity or cell wall modification. Limited pectin backbone depolymerisation has been reported for sweet cherry (Batisse et al., 1994), and ripening-related changes in firmness of these fruit have been attributed rather to the loss of sugar residues from pectin side-chains (Batisse et al. 1996; Salato et al. 2013). In accordance with this view, a β -galactosidase (β -Gal) purified from the ripe fruit of ‘Lapins’

sweet cherry was recently characterized, shown to increase activity along ripening, and suggested to play a key role in ripening-related softening of this fruit species (Gerardi et al. 2012). In an earlier study (Andrews and Li 1994), though, the highest β -Gal activity during on-tree development of 'Bing' fruit was found approximately two weeks before commercial maturity, pointing to an important role of the enzyme at early stages of the softening process. At any rate, a key role for β -galactosidase in firmness loss of sweet cherry fruit would involve that the loss of galactosyl residues from pectin side-chains is indeed a major event in pectin depolymerisation and solubilisation. Even so, firmness of 'Celeste' and 'Somerset' cherries after storage at 0 °C was found to correlate inversely with pectin methylesterase (PME) activity (Belge et al. 2015), which suggests that pectin backbone modifications are also involved in the softening process of these fruit.

Therefore, the activity of both pectin backbone- and pectin side chain-acting enzymes was examined, both at harvest and after storage. Pectate lyase (PL) activity levels increased slightly after storage, but no significant differences were found in response to treatment or to shelf life period at 20 °C (**Table 5**). Polygalacturonase (PG) activity increased noticeably after storage of fruit. Surprisingly, upon removal from cold storage, PG activity was 1.7 and 2.1 fold higher in samples treated with 1.5 and 3% CaCl_2 , respectively, with no apparent relationship to fruit firmness levels (**Table 2**). However, after three days at 20 °C the activity levels in untreated fruit were still higher, whereas those in calcium-treated samples decreased noticeably, the lowest activity corresponding to cherries treated with 1.5% CaCl_2 (**Table 5**), in agreement with higher fruit firmness.

PME activity levels may underlie these discrepancies between PG activity and fruit firmness. Indeed, PG cleaves demethylated D-galacturonic acid residues from pectin backbone galacturonans, and thus previous PME action is necessary for PG hydrolytic activity on its substrate (Goulao and Oliveira 2008). PME activity exerts a wide range of

major impacts on cell wall properties, as the removal of methyl moieties from D-galacturonic acid residues alters the charge and pH of the apoplast, and hence modifies the mobility and activity of other cell wall proteins. In this study, PG activity increased strongly after cold storage, even though fruit firmness was higher in comparison with levels at harvest (**Table 2**). However, PME activity levels were very low in cherries dipped in 3% CaCl₂ (**Table 5**). Low PME activity would prevent intensive demethylation of polyuronides, which would preclude PG action in spite of high activity levels present. As to samples treated in 1.5% CaCl₂, firmness was similar to that in untreated fruit, even though those fruit displayed higher activity levels for both PG and PME activities (**Table 5**). In the presence of exogenous calcium, high demethylating action by PME may have resulted in the establishment of additional calcium bridges throughout the pectin backbone rather than in facilitation of PG-catalysed cleavage of D-galacturonic acid residues. This idea is in accordance with the observation of significantly higher amounts of uronic acids retained in the CDTA-soluble fraction isolated from these fruit in comparison with the controls (**Fig. 1A**) in spite of similar firmness levels in both cases (**Table 2**). Data suggest furthermore that high (3%) calcium concentrations strongly inhibited PME activity. The reason for these discrepancies in comparison with the effects of the application of a lower dose (1.5%) is unclear. Additional pectin analyses, such as degree of methylation, would be of help in checking whether such differences actually resulted in stronger pectin demethylation in samples treated with 1.5% calcium. However, these differences in PME activity levels between both calcium treatments were found both 0 and 3 days after removal from cold storage (**Table 5**). A range of isoforms exist for virtually all cell wall-acting proteins, some of which may predominate under different conditions thus giving rise to apparently contradictory results. Primers for the RT-qPCR amplification of *PaPG1*, *PaPG2* and *PaPME* genes were designed, but unfortunately the amplification efficiencies were negligible, and thus the expression of these genes could

not be studied at the transcript level. Since primers were designed from gene sequences reportedly expressed in fruit tissues (accession numbers DQ659241, DQ659240 and AB231903) (Morgutti et al. 2006; Murayama et al. 2009), this observation may be indicating very low transcription rates for these genes.

Enzymes removing galactosyl- and arabinosyl-rich side chains in pectins affect cell wall porosity, and may thus modulate other cell wall activities by allowing or restricting the access to their substrates (reviewed in Goulao and Oliveira 2008). Limited changes were observed for α -L-arabinofuranosidase (AFase) activity, with apparently limited relationship to firmness changes, which contradicts previous findings for other fruit species such as apple (Ortiz et al. 2011c). However, the reportedly wide variation in the biochemical events leading to fruit softening among species, and even across cultivars within a given species, makes it advisable to avoid generalisations and to study this process on a case-by-case basis. The increase in fruit firmness upon removal from cold storage (**Table 2**), though, was accompanied by lower β -galactosidase activity levels (**Table 5**), which rose again after fruit were left for three days at 20 °C, coincident with the lowest firmness levels. This is consistent with the observation of lowered contents of neutral sugars in the Na₂CO₃-soluble fraction (**Fig. 1B**), and suggests that significant loss of galactosyl residues from pectins was taking place during the softening process, in accordance with previous suggestions that β -Gal may play a key role in ripening-related softening of sweet cherry fruit (Gerardi et al. 2012). The observed β -Gal activity values generally, albeit not totally, agreed with the corresponding gene expression levels (**Fig. 2**): cold storage appeared to inhibit the relative expression of a putative *Pa β -Gal* gene, to recover to similar levels to those at harvest after three days at 20 °C. Again, it should be stressed that only one *β -Gal* gene was studied, and that additional homologous genes may be contributing to the total β -Gal activity detected in the enzyme assays.

On the basis of some previous experimental evidence that firmness of sweet cherry fruit may also relate in part to the amount and structure of xyloglucans (Choi et al. 2002), some non-pectolytic proteins were considered as well. Matrix glycans are susceptible to hydrolytic cleavage by β -xylosidase (β -Xyl), which removes successive D-xylosyl residues from the non-reducing end. In this study, calcium inhibited β -Xyl activity during cold storage, but caused a sharp increase after shelf life while, in contrast, β -Xyl activity in untreated fruit was unaffected during cold storage to decrease significantly after fruit were kept at 20 °C for three days (**Table 5**), in agreement with previous observations for apple fruit (Ortiz et al. 2011c). Xyloglucans can be cleaved also by the action of endo-1,4- β -D-glucanase (EGase). Similarly to observations for β -Xyl, EGase activity was inhibited during cold storage in calcium-treated fruit, but not thereafter (**Table 5**), albeit in this case no significant differences were observed between untreated and treated fruit. Gene expression data were in accordance with those for enzyme activity: cold storage increased *PaEGase* expression rates as compared with levels at harvest in untreated fruit, but not in calcium-treated samples, while levels equalled again after the shelf life period at 20 °C regardless of treatment (**Fig. 2**). Yet, a discrepancy was found: *PaEGase* gene expression increased sharply in H+3 fruit in comparison with rates at harvest, while no significant differences were observed regarding activity levels (**Table 5**). Again, results illustrate the complexity of cell wall disassembly-associated phenomena, and the difficulty of relating particular events to changes observed in the expression or activity of related genes and enzymes. Other hemicellulose-acting enzymes such as xyloglucan endotransglycosylase/hydrolases (XTH) were not studied at the activity level, but the relative expression of a *PaXTH* gene was assessed (**Fig. 2**). Interestingly, expression levels were strongly inhibited during cold storage in untreated samples, but only marginally in calcium-treated fruit, and remained at levels close to those at harvest after three days at 20 °C irrespective of treatment. Actually, XTH activity levels remained unchanged during ripening

of ‘Mondial Gala’ apples (Goulao et al. 2007), even though differences in the transcription rates of two individual *XTH* genes were detected (Goulao et al. 2008). In this work, though, decreased expression rates for *PaXTH* coincided with increased firmness levels in H+3 and cold-stored control fruit (**Table 2**). However, the highest firmness values were observed for calcium-treated fruit upon removal from refrigerated storage, in spite of the fact that *PaXTH* gene expression was also higher than that in the controls (**Fig. 2**). XTH enzymes are considered to help preparing the cell wall for modification through the action of other related enzymes, both by loosening the structure and by direct xyloglucan disassembly. But, similarly to PME, XTH exerts a dual role in cell wall metabolism: although it hydrolyses xyloglucans (XEH activity), it can also act as a transglucosylase (XET activity) which can integrate *de novo*-secreted xyloglucans into the already existing xyloglucan network or restructure xyloglucan molecules (Goulao et al. 2008). Many *XTH* isogenes have been identified, and for apple and tomato fruit they have been demonstrated to be under ethylene control (Muñoz-Bertomeu et al. 2013). The question arises, though, as to how *XTH* expression and XTH activity are regulated in a non-climacteric fruit such as sweet cherry.

3.3. A cross-talk between enzymatic and non-enzymatic effectors in cell wall changes?

Expansins are non-enzymatic proteins which contribute importantly to the loosening of hydrogen bonds between cell wall polymers, and over recent years they have been receiving ever more research focus as a possible major driver of firmness loss in fruit commodities. Expansins belong to large multigene families, but *EXPI* is reportedly the major ripening-associated expansin gene in tomato fruit (Brummell et al. 1999). EXP1-suppressed tomato fruit were firmer, while fruit were much softer when the gene was overexpressed (Brummell et al. 1999). Three expansin genes, *PpEXP1*, *PpEXP2* and *PpEXP3*, have been reported to be up-regulated at the onset of ripening of peach fruit (Hayama et al. 2000, 2003), but *PpEXP1* was

induced at an earlier maturity stage, whereas *PpEXP3* was suggested to be related to the changes in fruit firmness. In this work, primers were designed for the RT-qPCR amplification of two expansin genes, namely *PaEXPI* and *PaEXP3*. No amplification was detectable for *PaEXP3*, but the expression levels for *PaEXPI* in fruit pericarp in relation to those at harvest are shown (**Fig. 2**). Cold storage resulted in a sharp decline in *PaEXPI* gene expression, which however recovered to a great extent during the shelf life period, possibly in connection with lowered fruit firmness (**Table 2**). Even so, expression levels after three days at 20 °C were higher in calcium-treated as compared with untreated fruit (**Fig. 2**). If calcium treatment actually helped delaying firmness loss of ‘Celeste’ cherries, this observation would be consistent with the reported early induction of the *EXPI* isogene during ripening of peach fruit (Hayama et al. 2003).

Non-enzymatic modulation of cell wall changes might also be exerted through the control of the apoplastic redox status. Ascorbic acid (AA) is the main antioxidant buffer in the apoplast, where the external stimuli are rapidly perceived thus leading to alterations in its micro-environment (Pignocchi and Foyer 2003). Early during the ripening process, cell membrane permeability increases, which event allows the release of ascorbate into the apoplast, where hydroxyl ($\cdot\text{OH}$) radicals are generated in the presence of O_2 and Cu^{2+} (Fry 1998). In addition, experimental evidence exists that D-galacturonic acid released from cell walls can be a major precursor for the biosynthesis of L-ascorbic acid in fruits (Agius et al. 2003). In this work, ascorbate release into the apoplast was apparently stimulated in calcium-treated fruit after refrigerated storage, as AA levels measured in lyophilised fruit pericarp, corresponding mainly to cytosolic ascorbate, were significantly lower in comparison with those both at harvest and the controls, while concomitantly increasing in the insoluble cell wall materials (**Table 3**). Contrarily, no significant differences in the ascorbate content in the pericarp were found between treated and untreated fruit after the shelf life period, whereas

those in the cell wall materials were lower in treated fruit in comparison with the controls. AA can be oxidised to dehydroascorbic acid (DHA), which must be returned to the cytosol for recycling to AA. Cell wall-localised ascorbate oxidase (AO) activity can lead to decreased abundance of Ca^{2+} -channel transcripts through apoplast oxidation (Pignocchi et al. 2006). Increases in apoplastic DHA favour cell wall loosening, which would provide an additional mechanism for cell wall disassembly. It has been suggested that DHA may react with lysine and arginine residues and hence prevent cross-linking of structural proteins with cell wall polysaccharides (Lin and Varner 1991). DHA may also be converted to oxalate, and the formation of calcium oxalate crystals would lower the levels of ionic calcium and thus hinder the establishment of calcium bridges between polyuronides. It has been also demonstrated that both AA and DHA can induce oxidative scission of plant cell wall polysaccharides at physiological pH ranges, xyloglucan showing higher susceptibility to ascorbate-induced scission than pectic polymers (Fry 1998). Interestingly, it was later found out that de-esterified citrus pectin is more susceptible to ascorbate-induced scission *in vitro* than methyl-esterified pectin (Dumville and Fry 2003), thus pointing to an additional role for PME activity in ripening-related softening.

Results reported herein suggest an important role for PME, β -Gal and expansin action in ripening-related softening of ‘Celeste’ sweet cherry fruit, exerted possibly through the modulation of the access of additional proteins to their substrates. Data are also consistent with a role for the redox status of the apoplast in this process. Calcium dips delayed firmness loss of fruit after cold storage and the subsequent shelf life at 20 °C. The combination of both procedures was also effective in decreasing decay and weight loss.

Acknowledgments

B. Belge was the recipient of a FI-DGR grant from AGAUR (Generalitat de Catalunya). This work was supported through the AGL2010-14801/ALI project, funded by the Ministerio de Ciencia e Innovación (MICINN) of Spain. Gene expression analyses were undertaken in L.F. Goulao's laboratory thanks to a short-term scholarship granted to B. Belge by the University of Lleida. We are indebted to Mrs. Vânia Cardoso for invaluable technical assistance. Fruit samples were provided by J.M. Camats (Cireres de Corbins Camats-Carpi).

Conflict of interest

The authors declare no potential conflicts of interest.

References

- Agius, F., González-Lamothe, R., Caballero, J.L., Muñoz-Blanco, J., Botella, M.A., Valpuesta, V., 2003. Engineering increased vitamin C levels in plants by overexpression of a D-galacturonic acid reductase. *Nat. Biotechnol.* 21, 177-181.
- Alkio, M., Jonas, U., Sprink, T., van Nocker, S., Knoche, M., 2012. Identification of putative candidate genes involved in cuticle formation in *Prunus avium* (sweet cherry) fruit. *Ann. Bot.* 110, 101-112.
- Andrews, P.K., Li, S., 1994. Partial purification and characterization of β -D-Galactosidase from sweet cherry, a nonclimacteric fruit. *J. Agric. Food Chem.* 42, 2177-2182.
- Batisse, C., Buret, M., Coulomb, P.J., 1996. Biochemical differences in cell wall of cherry fruit between soft and crisp fruit. *J. Agric. Food Chem.* 44, 453-457.
- Batisse, C., Fils-Lycaon, B., Buret, M., 1994. Pectin changes in ripening cherry fruit. *J. Food Sci.* 59, 389-393.

- Belge, B., Comabella, E., Graell, J., Lara, I. 2015. Post-storage cell wall metabolism in two sweet cherry (*Prunus avium* L.) cultivars displaying different postharvest performance. Food Sci. Technol. Int. 21, 416-427.
- Bian, W.H., Jiang, C.J., Yu, Y.B., Wang, Z.X., Wan, X.C., 2005. *Prunus persica* beta-galactosidase mRNA, complete cds. NCBI Database, GenBank: AY874412.1.
- Botton, A., Tonutti, P., 2002. *Pp-XET1*: a putative gene coding for xyloglucan-endotransglycosylase 1 of peach (*Prunus persica* L. Batsch). NCBI Database, GenBank: AF534870.1.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72, 248-254.
- Brummell, D.A., Harpster, M.H., Civello, P.M., Palys, J.M., Bennett, A.B., Dunsmuir, P., 1999. Modification of expansin protein abundance in tomato fruit alters softening and cell wall polymer metabolism during ripening. Plant Cell 11, 2203-2216.
- Brummell, D.A., Howie, W.J., Ma, C., Dunsmuir, P., 2002. Postharvest fruit quality of transgenic tomatoes suppressed in expression of a ripening-related expansin. Postharvest Biol. Technol. 25, 209-220.
- Chang, S., Puryear, J., Cairney, J., 1993. A simple and efficient method for isolating RNA from pine trees. Plant Mol. Biol. Rep. 11, 113–116.
- Choi, C., Toivonen, P., Wiersma, P.A., Kappel, F., 2002. Differences in levels of pectic substances and firmness in fruit from six sweet cherry genotypes. J. Am. Pomol. Soc. 56, 197–201.
- Civello, P.M., Powell, A.L.T., Sabehat, A., Bennett, A.B., 1999. An expansin gene expressed in ripening strawberry fruit. Plant Physiol. 121: 1273–1279.

530 Demirsoy, L.K., Bilgener, S., 1998. The effects of preharvest calcium hydroxide applications
 531 on cracking in '0900 Ziraat', 'Lambert' and 'Van' sweet cherries. *Acta Hort.* 468, 657-
 532 662.

533 Dumville, J.C., Fry, S.C., 2003. Solubilisation of tomato fruit pectins by ascorbate: a possible
 534 non-enzymic mechanism of fruit softening. *Planta* 217, 951-961.

535 Fry, S.F., 1998. Oxidative scission of plant cell wall polysaccharides by ascorbate-induced
 536 hydroxyl radicals. *Biochem. J.* 332, 507-515.

537 Gerardi, C., Blando, F., Santino, A., 2012. Purification and chemical characterisation of a cell
 538 wall-associated β -galactosidase from mature sweet cherry (*Prunus avium* L.) fruit. *Plant*
 539 *Physiol. Biochem.* 61, 123-130.

540 Gillespie, K.M., Ainsworth, E.A., 2007. Measurement of reduced, oxidized and total
 541 ascorbate content in plants. *Nat. Protoc.* 2, 871-874.

542 Goulao, L.F., Cosgrove, D.J., Oliveira, C.M., 2008. Cloning, characterisation and expression
 543 analyses of cDNA clones encoding cell wall-modifying enzymes isolated from ripe
 544 apples. *Postharvest Biol. Technol.* 48, 37-51.

545 Goulao, L.F., Oliveira, M.C., 2008. Cell wall modifications during fruit ripening: when a fruit
 546 is not the fruit. *Trends Food Sci. Technol.* 19, 4-25.

547 Goulao, L.F., Santos, J., de Sousa, I., Oliveira, C.M., 2007. Patterns of enzymatic activity of
 548 cell wall-modifying enzymes during growth and ripening of apples. *Postharvest Biol.*
 549 *Technol.* 43, 307-318.

550 Goulao, L.F., Fortunato, A.S., Ramalho, J.C., 2012. Selection of reference genes for
 551 normalizing quantitative real-time PCR gene expression data with multiple variables in
 552 *Coffea* spp. *Plant Mol. Biol. Rep.* 30, 741-759.

553 Harrison, E.P., McQueen-Mason, S.J., Manning, K., 2001. Expression of six expansin genes
 554 in relation to extension activity in developing strawberry fruit. *J. Exp. Bot.* 52: 1437–
 555 1446.

556 Hayama, H., Shimada, T., Haji, T., Ito, A., Kashimura, Y., Yoshioka, H., 2000. Molecular
 557 cloning of a ripening-related expansin cDNA in peach: evidence for no relationship
 558 between expansin accumulation and change in fruit firmness during storage. *J. Plant*
 559 *Physiol.* 157: 567–573.

560 Hayama, H., Ito, A., Moriguchi, T., Kashimura, Y., 2003. Identification of a new expansin
 561 gene closely associated with peach fruit softening. *Postharvest Biol. Technol.* 29, 1-10.

562 Lara, I., 2013. Preharvest sprays and their effects on the postharvest quality of fruit. *Stewart*
 563 *Postharvest Review* 3, 5.

564 Lara, I., García, P., Vendrell, M., 2004. Modifications in cell wall composition after cold
 565 storage of calcium-treated strawberry (*Fragaria × ananassa* Duch.) fruit. *Postharvest*
 566 *Biol. Technol.* 34, 331-339.

567 Lin, L.S., Varner, J.E., 1991. Expression of ascorbic acid oxidase in zucchini squash
 568 (*Cucurbita pepo* L.). *Plant Physiol.* 96, 159-165.

569 Morgutti, S., Negrini, N., Nocito, F.F., Ghiani, A., Bassi, D., Cocucci, M., 2006. Changes in
 570 endopolygalacturonase levels and characterization of a putative *endo-PG* gene during
 571 fruit softening in peach genotypes with nonmelting and melting flesh fruit phenotypes
 572 *New Phytol.* 171, 315-328.

573 Muñoz-Bertomeu, J., Miedes, E., Lorences, E.P., 2013. Expression of xyloglucan
 574 endotransglucosylase/hydrolase (*XTH*) genes and XET activity in ethylene treated apple
 575 and tomato fruits. *J. Plant Physiol.* 170, 1194-1201.

576 Murayama, H., Arikawa, M., Sasaki, Y., Dal Cin, V., Mitsuhashi, W., Toyomasu, T., 2009.
 577 Effect of ethylene treatment on expression of polyuronide-modifying genes and

solubilization of polyuronides during ripening in two peach cultivars having different softening characteristics. *Postharvest Biol. Technol.* 52, 196-201.

Ortiz, A., Graell, J., Lara, I., 2011a. Preharvest calcium applications inhibit some cell wall-modifying enzyme activities and delay cell wall disassembly at commercial harvest of ‘Fuji Kiku-8’ apples. *Postharvest Biol. Technol.* 62, 161–167.

Ortiz, A., Vendrell, M., Lara, I., 2011b. Softening and cell wall metabolism in late-season peach in response to controlled atmosphere and 1-MCP treatment. *J. Hortic. Sci. Biotech.* 86, 175-181.

Ortiz, A., Graell, J., Lara, I., 2011c. Cell wall-modifying enzymes and firmness loss in ripening ‘Golden Reinders’ apples: A comparison between calcium dips and ULO storage. *Food Chem.* 128, 1072-1079.

Pignocchi, C., Foyer, C.H., 2003. Apoplastic ascorbate metabolism and its role in the regulation of cell signalling. *Curr. Opin. Plant Biol.* 6, 379-389.

Pignocchi, C., Kiddle, G., Hernández, I., Foster, S.J., Asensi, A., Taybi, T., Barnes, J., Foyer, C.H., 2006. Ascorbate oxidase-dependent changes in the redox state of the apoplast modulate gene transcript accumulation leading to modified hormone signalling and orchestration of defense processes in tobacco. *Plant Physiol.* 141, 423-435.

Redgwell, R.J., Melton, L.D., Brasch, D.J., 1992. Cell wall dissolution in ripening kiwifruit (*Actinidia deliciosa*). *Plant Physiol.* 98, 71-81.

Salato, G.S., Ponce, N.M.A., Raffo, M.D., Vicente, A.R., Stortz, C.A., 2013. Developmental changes in cell wall polysaccharides from sweet cherry (*Prunus avium* L.) cultivars with contrasting firmness. *Postharvest Biol. Technol.* 84, 66-73.

Selvendran, R.R., O’Neill, M.A., 1987. Isolation and analysis of cell walls from plant material. In: Glick, D. (Ed.), *Methods of Biochemical Analysis*, vol. 32. John Wiley Interscience, New York, pp. 25-153.

603 Trainotti, L., Spolaore, S., Ferrarese, L., Casadoro, G., 1997. Characterization of PpEG1, a
 604 member of a multigene family which encodes endo-beta-1,4-glucanase in peach. *Plant*
 605 *Mol. Biol.* 34, 791-802.

606 Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman,
 607 F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric
 608 averaging of multiple internal control genes. *Genome Biol.* 3, research0034.1-0034.11.

609 Vangdal, E., Hovland, K.L., Børve, J., Sekse, L., Slimestad, R., 2008. Foliar application of
 610 calcium reduces postharvest decay in sweet cherry by various mechanisms. *Acta Hort.*
 611 768, 143-148.

612 Wang, Y., Xie, X.B., Long, L.E., 2014. The effect of postharvest calcium application in
 613 hydro-cooling water on tissue calcium content, biochemical changes, and quality
 614 attributes of sweet cherry fruit. *Food Chem.* 160, 22-30.

Table 1.

Primers used for RT-qPCR analysis of cell wall-related gene expression in ‘Celeste’ sweet cherry fruit at harvest and after cold storage following a calcium treatment.

Gene ^a	Accession no.	Primers	Amplicon (bp)	Reference
<i>Paβ-Gal</i>	AY874412.1	F: 5'-CTGTGGGAGCTACCAAGAAGG-3' R: 5'-ATGGTGACTGCACATCTCTGC-3'	96	Bian et al., 2005
<i>PaEG</i>	X96853.1	F: 5'-GATTGAGAATGCCAAGGATGC-3' R: 5'-GTTTGGATCTGCCACTTGGAC-3'	103	Trainotti et al., 1997
<i>PaXHT</i>	AF534870.1	F: 5'-GCTTGTGAAGGCAGATTGGAG-3' R: 5'-AGCCTGAAGACCAAGTGCAAG-3'	86	Botton and Tonutti, 2002
<i>PaEXP1</i>	AB029083.1	F: 5'-CATTTTATGGTGGGGGTGATG-3' R: 5'-GAGAGCTGCAGTGTGGTTCC-3'	98	Hayama et al., 2003
Reference gene ^b				
<i>PaCAC</i>	JU090715.1	F: 5'-CTCCGCACTTCCTTTTGGTAC-3' R: 5'-ATCTCGCAGTTCTTCGTGCTC-3'	87	Alkio et al., 2012
<i>PaPP2A-2</i>	JU090712.1	F: 5'-TCATCAGCTTGTCATGGAAGG-3' R: 5'-ATTGCAGCCATATTCCCACAC-3'	105	Alkio et al., 2012
<i>PaSRP19</i>	JU090733.1	F: 5'-GGGGAGGTCCTTATCCATGTC-3' R: 5'-GCCACCTAGCGCATTGTATTTC-3'	111	Alkio et al., 2012

^a *Paβ-Gal*, β-galactosidase; *PaEG*, endo-1,4-β-D-glucanase; *PaXHT*, xyloglucan endotransglycosylase/hydrolase; *PaEXP1*, expansin.

^b *PaCAC*, Clathrin adaptor complexes protein; *PaPP2A-2*, Protein phosphatase 2A-2; *PaSRP19*, Signal recognition particle SRP19.

Table 2. Maturity and quality attributes of ‘Celeste’ sweet cherry fruit at harvest and after cold storage following a calcium treatment.

Parameter	H ^a	H+3	14 + 0 ^b			14 + 3		
			UT ^c	1.5%	3%	UT	1.5%	3%
Respiration ($\mu\text{g CO}_2 \text{ kg}^{-1} \text{ s}^{-1}$)	107 c	82 d	175 b	208 a	212 a	78 d	82 d	87 d
Weight loss (%)	-	11.9 c	6.8 d	6.9 d	6.2 e	14.4 a	14.3 a	13.8 b
Size ^d (mm)	27.7 a	24.6 e	26.0 bc	25.8 c	26.3 b	24.8 e	25.1 d	24.9 de
Juiciness (mL kg ⁻¹)	544 a	507 c	549 a	532 b	553 a	408 f	454 e	485 d
Firmness (Durofel units)	74.9 c	79.1 b	80.5 b	79.1 b	83.0 a	66.4 e	75.2 c	69.6 d
SSC (%, w/w)	17.2 b	19.3 a	16.4 c	16.0 c	17.9 b	17.2 b	17.3 b	17.6 b
Acidity (%, w/w) ^e	0.9 b	1.0 a	0.8 c	0.7 d	0.8 c	0.8 c	0.8 c	0.8 c
SSC/TA ratio	1.9	2.0	2.1	2.2	2.4	2.1	2.1	2.2
Decay (%)	-	16.1 b	22.2 a	12.1 c	7.5 d	23.9 a	12.8 c	14.2 bc

Firmness, size and decay values are the average of 30 individual fruit. The rest of values represent means of three replicates of 10 fruit each. Means followed by different letters within the same row are significantly different at $P \leq 0.05$ (LSD test).

^a At harvest; ^b Days at 0 °C + days at 20 °C; ^c Untreated; ^d Size is given as the average between the wider and the narrower sides of each fruit; ^e Acidity is expressed as equivalents of malic acid.

Table 3. Content of calcium (g kg^{-1}), and of reduced (AA) and oxidised (DHA) ascorbic acid (mmol kg^{-1}) in lyophilised pericarp tissue and in the insoluble cell wall materials recovered from ‘Celeste’ sweet cherry fruit at harvest and after cold storage following a calcium treatment.

Ca^{2+}	H^a	H+3	14 + 0^b			14 + 3		
			UT^c	1.5%	3%	UT	1.5%	3%
Pericarp	0.45 c	0.35 c	0.41 c	0.77 ab	0.85 a	0.37 c	0.72 b	0.75 ab
Cell wall	1.85 d	1.65 d	2.25 c	4.07 a	3.41 b	2.25 c	3.62 b	3.70 b

AA

Pericarp	19.25 b	23.27 a	20.04 b	14.39 d	15.95 c	15.46 c	14.65 cd	15.57 c
Cell wall	1.48 d	1.62 cd	1.38 d	3.43 a	3.45 a	3.38 a	2.79 b	1.84 c

DHA

Pericarp	n.d.	n.d.	n.d.	n.d.	0.55 b	0.56 b	1.35 a	1.94 a
Cell wall	n.d.	n.d.	n.d.	n.d.	n.d.	0.36 a	0.10 b	n.d.

Values are the means of three replicates (n.d., non-detectable). Means followed by different letters within the same row are significantly different at $P \leq 0.05$ (LSD test).

^a At harvest; ^b Days at 0 °C + days at 20 °C; ^c Untreated.

Table 4. Yield (g kg⁻¹) of insoluble cell wall materials (CWM) and of cell wall fractions expressed over lyophilised pericarp tissue (**A**) and over total isolated insoluble cell wall materials (CMW) (**B**) of ‘Celeste’ sweet cherry fruit at harvest and after cold storage following a calcium treatment.

A								
Fraction	H ^a	H+3	14 + 0 ^b			14 + 3		
			UT ^c	1.5%	3%	UT	1.5%	3%
Total CWM	44.0 c	56.9 b	58.5 b	67.4 a	57.3 b	70.1 a	58.4 b	67.8 a
Water-soluble	3.1 a	2.8 a	1.7 b	1.5 b	1.4 b	1.7 b	0.8 c	2.4 ab
CDTA-soluble	5.4 d	9.1 c	6.2 d	12.4 b	13.5 b	14.2 b	13.5 b	17.1 a
Na₂CO₃-soluble	3.4 c	3.2 c	6.1 b	7.7 a	5.4 b	6.1 b	7.2 a	6.4 ab
KOH-soluble	0.4 b	0.6 ab	0.7 a	0.6 ab	0.6 ab	0.3 b	0.7 a	0.7 a

B								
Fraction	H ^a	H+3	14 + 0 ^b			14 + 3		
			UT ^c	1.5%	3%	UT	1.5%	3%
Water-soluble	70.6 a	48.8 b	28.5 d	22.9 d	25.1 d	24.5 d	13.4 e	36.0 c
CDTA-soluble	121.8 cd	159.7 c	106.6 d	183.9 b	234.9 ab	202.4 b	230.4 ab	252.8 a
Na₂CO₃-soluble	77.1 f	56.2 g	104.8 c	113.8 b	95.0 d	86.3 e	123.4 a	94.1 d
KOH-soluble	9.3 b	10.0 bc	11.6 a	8.3 c	9.9 bc	3.9 d	11.4 a	10.9 a

Values are the means of three replicates. Means followed by different letters within the same row are significantly different at $P \leq 0.05$ (LSD test).

^a At harvest; ^b Days at 0 °C + days at 20 °C; ^c Untreated.

Table 5. Specific activity (U 10⁶ kg⁻¹) of some pectolytic and non-pectolytic enzymes in the pericarp tissue of ‘Celeste’ sweet cherry fruit at harvest and after cold storage following a calcium treatment.

Pectolytic	H ^a	H+3	14 + 0 ^b			14 + 3		
			UT ^c	1.5%	3%	UT	1.5%	3%
Poligalacturonase (PG)	25.58 e	24.92 e	35.51 d	60.71 b	74.41 a	57.98 bc	39.12 d	50.55 c
Pectate lyase (PL)	1.56 b	1.89 b	2.04 ab	2.20 ab	2.36 a	2.37 a	2.09 ab	2.33 a
Pectin methylesterase (PME)	28.89 c	45.15 ab	18.86 d	52.21 a	4.66 e	31.63 b	48.54 ab	29.39 c
β-galactosidase (β-Gal)	10.78 b	10.10 b	9.9 bc	8.06 d	9.21 c	10.33 b	9.75 bc	11.20 a
α-L-arabinofuranosidase (AFase)	0.25 c	0.30 a	0.28 b	0.26 c	0.29 ab	0.29 ab	0.28 b	0.30 a

Non-pectolytic								
endo-1,4-β-D-glucanase (EGase)	4.00 c	4.68 bc	5.34 a	5.00 b	4.93 b	5.83 a	3.84 c	5.62 a
β-xylosidase (β-Xyl)	70.50 c	55.35 e	71.04 c	63.39 d	62.81 d	61.85 d	76.56 b	90.54 a

Values are the means of three replicates. Means followed by different letters within the same row are significantly different at $P \leq 0.05$ (LSD test).

^a At harvest; ^b Days at 0 °C + days at 20 °C; ^c Untreated.

Figure captions

Figure 1. Amount (g kg^{-1} DW) of neutral sugars and uronic acids in the CDTA- (**A**) and the Na_2CO_3 -soluble (**B**) fractions isolated from the pericarp tissue of ‘Celeste’ sweet cherry fruit at harvest (H) and after cold storage following a calcium treatment (UT, untreated). Values are means of three replicates. Different letters represent significant differences in the total amount of uronic acids and neutral sugars in each of both pectin-containing fractions at $P \leq 0.05$ (LSD test).

Figure 2. Relative expression of some cell-wall related genes in the pericarp tissue of ‘Celeste’ sweet cherry fruit 3 d after harvest (H+3) and after cold storage following a calcium treatment (UT, untreated; X+Y, days at 0 °C + days at 20 °C, respectively). Expression level values represent means of three replicates. For a given gene, different letters denote significant differences at $P \leq 0.05$ (LSD test).

Supplementary Figures

Figure S1. Relative percentage (g kg^{-1}) of neutral sugars and uronic acids in the CDTA- (A) and the Na_2CO_3 -soluble (B) fractions isolated from the pericarp tissue of ‘Celeste’ sweet cherry fruit at harvest (H) and after cold storage following a calcium treatment (UT, untreated). Values are means of three replicates. Different letters represent significant differences in the total amount of uronic acids and neutral sugars in each of both pectin-containing fractions at $P \leq 0.05$ (LSD test).

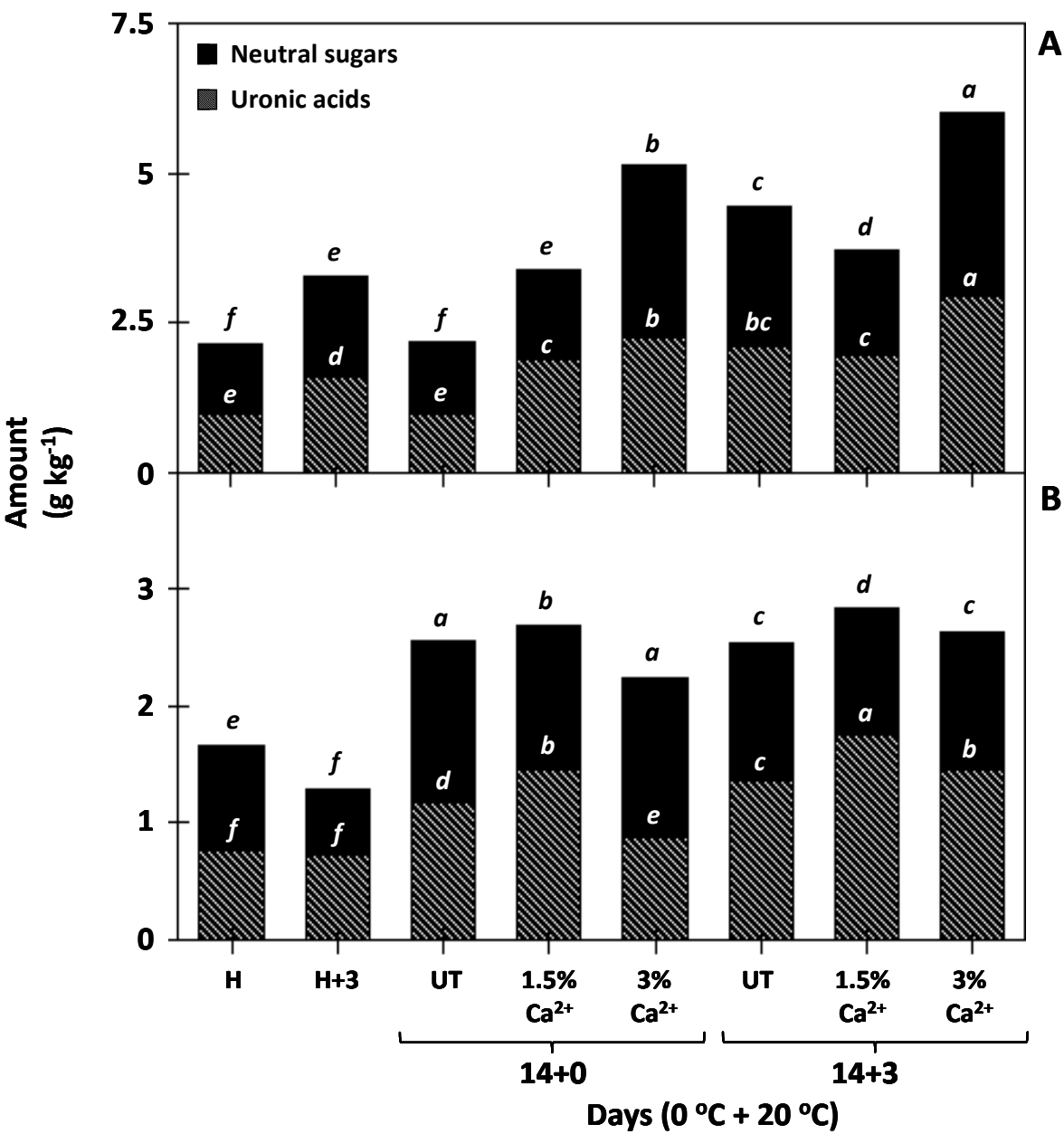


Figure 1

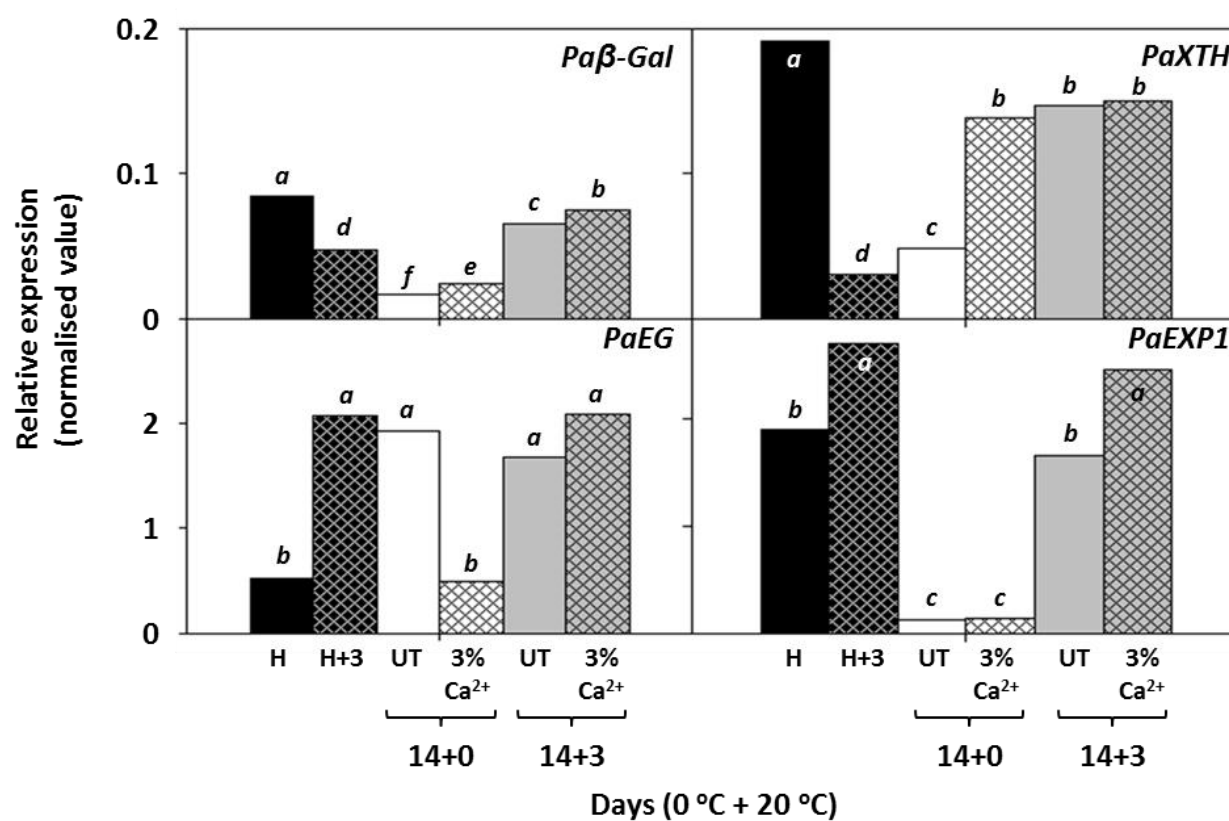


Figure 2

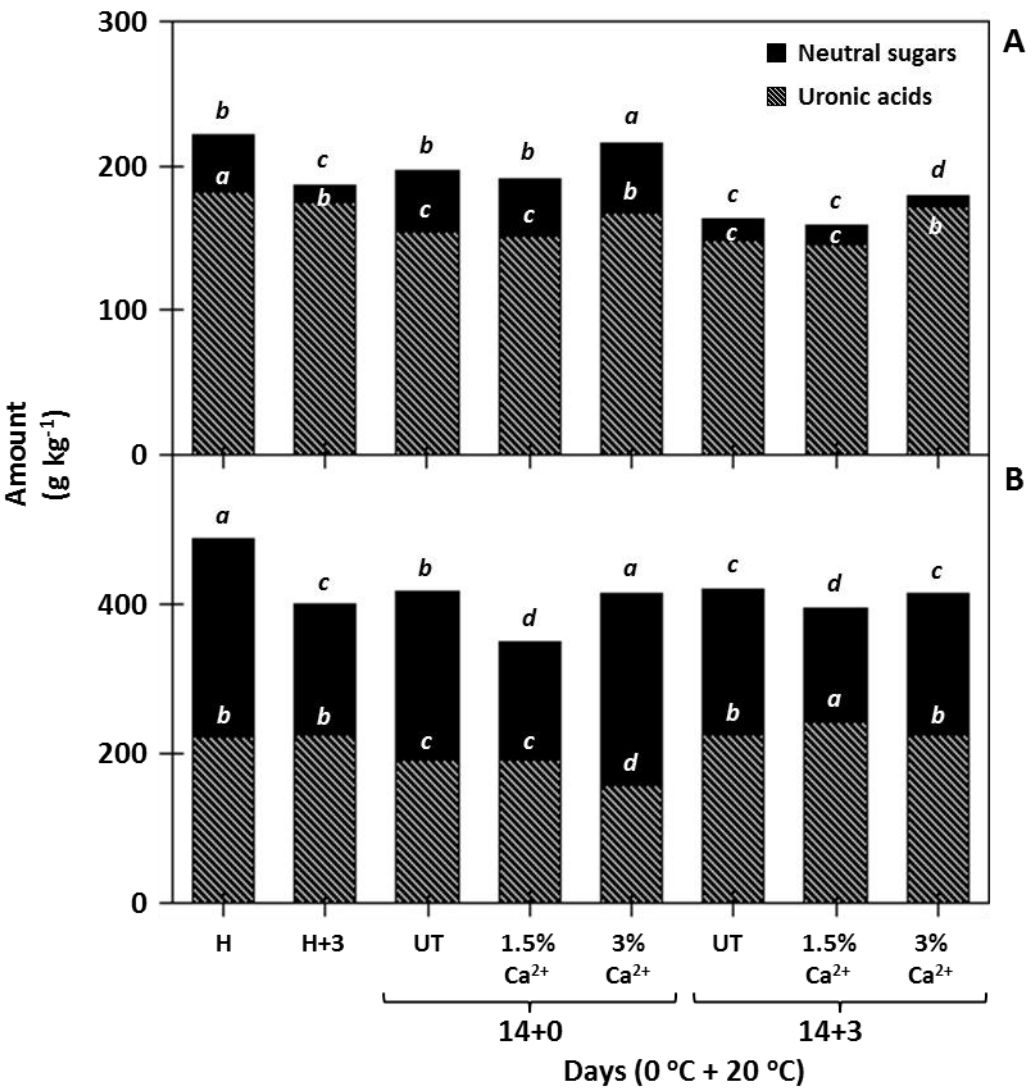


Figure S1